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Effects of calmodulin antagonists and anesthetics on the skin lesions induced by 2-chloroethylethyl sulfide

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Abstract

The effects of calmodulin antagonists and anesthetics on the skin lesions induced by an alkylating vesicant, 2-chloroethylethyl sulfide, were investigated using female hairless mice. 2-Chloroethylethyl sulfide, topically applied (0.6 μl/5 mm in diameter) on the back skin of hairless mice, induced mild to moderate petechiae on the 1st day, and ulcers with a thick scab after 3 days. The healing process started after 6 days, resulting in shedding of scabs on 9.52 days. Water-soluble ointment bases showed some beneficial effects, whereas oily bases made the skin lesions worse. Trifluoperazine (0.5–1%) and thioridazine (2%), potent calmodulin antagonists, in Pluronic F-127 base substantially prevented the development of 2-chloroethylethyl sulfide-induced skin lesions. A similar effect was achieved with pentamidine (10%), another type of calmodulin antagonist, but not with ketoconazole, a weak calmodulin antagonist. In addition, anesthetics, such as lidocaine and pentobarbital, showed some protection, although at high concentrations (> 5%). As judged by the microscopic appearance, trifluoperazine successfully reduced the hemorrhage and the infiltration of inflammatory cells in early skin lesions, and the formation of thick scabs, which leads to granulomatous scar tissue in late lesions. These results suggest that some calmodulin antagonists and anesthetics in water-soluble bases might be a choice for the treatment of 2-chloroethylethyl sulfide-induced skin burns.

Keywords: 2-Chloroethylethyl sulfide; Skin lesion, burn; Calmodulin antagonist; Anesthetic

1. Introduction

Recently, interest in the prevention or the therapy of skin damages induced by alkylating agents, such as 2,2′-dichlorodiethyl sulfide and 2-chloroethylethyl sulfide, has increased. However, there are no satisfactory prophylactic or therapeutic measures available. Although it is known that these vesicating compounds are mutagenic, carcinogenic and cytotoxic (Wormser, 1991), the biochemical mechanisms for tissue injury are not clearly understood. In earlier reports, a mechanism of toxicity was proposed based on the finding that sulfur mustards cause DNA strands to break, leading to the activation of poly(ADP-ribose) polymerase, and eventually to the depletion of intracellular NAD and ATP. Energy depletion could be responsible for cell death (Meier et al., 1987) and tissue injury (Papirmeister et al., 1985) by activating and releasing degradation enzymes. Additional cytotoxic mechanisms have been proposed: (a) glutathione depletion, resulting in lowered Ca^{2+}-ATPase activity and thereby disturbances of intracellular Ca^{2+} homeostasis, followed by the release of arachidonic acid from membranes by Ca^{2+}-phospholipase A_2 (Gentilhomme et al., 1992; Hua et al., 1993; Ray et al., 1995); (b) oxidative stress (Elsayed et al., 1989, 1992); and (c) lysosomal labilization (Choi et al., 1995; Shin et al., 1995). In spite of the protective potential of inhibitors of the above mechanisms against mustard cytotoxicity (Meier and Johnson, 1992; Choi et al., 1995),

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no good correlation between cell culture systems and skin tests has been shown (Mol et al., 1991).

Meanwhile, it is known that the gross lesions of skin exposed to mustards are very much similar to those of thermal burn, which shows erythema, edema, necrosis and ulcer (Vogt et al., 1984; Requena et al., 1988; Yourick et al., 1993). Moreover, the development of typical blisters, separation of dermal-epidermal junctions, is common in the skin lesions induced by thermal and mustard burns in human and some animal species.

Recently, calmodulin antagonists, such as trifluoperazine and CGS 9343B, were found to be potentially effective in the prevention of biochemical and pathological changes induced by thermal burn (Beitner et al., 1989b,1991) or frostbite (Beitner et al., 1989a). Those calmodulin antagonists may act by either binding to the Ca^{2+}-calmodulin complex or rendering it biologically inert with regard to the activation of Ca^{2+}-phospholipase A_2 and glucose 1,6-bisphosphatase. It is worthwhile to notice that Ca^{2+}-phospholipase A_2 may be involved commonly in mustard and thermal burns. In this context, we investigated whether compounds that have calmodulin antagonist activity exhibit such a beneficial effect on 2-chloroethylethyl sulfide-induced burns of hairless mouse skin. In addition, the effect of local anesthetics, such as lidocaine, tetracaine and butacaine, which are known to inhibit Ca^{2+}-phospholipase A_2, was also examined.

2. Materials and methods

2.1. Materials

2-Chloroethylethyl sulfide was provided by Aldrich Chemical (Milwakee, WI, USA), and calmodulin antagonists and Pluronic F-127 were procured from Sigma Chemical (St. Louis, MO, USA). The various ingredients of the ointment bases were obtained from commercial sources.

2.2. Preparation of ointments

Ointment bases were formulated as follows (%):

1. Hydrophobic base: white petrolatum (64.0), purified lanolin (16.0), light mineral oil (20.0)
2. W/o base: purified lanolin (70.0), distilled water (30.0)
3. O/w base: sodium lauryl sulfate (1.0), propylene glycol (12.0), stearyl alcohol (25.0), white petrolatum (25.0), cremophor RH40 (0.4), distilled water (36.6)
4. Polyethylene glycol base: polyethylene glycol 300 (33.0), polyethylene glycol 1500 (22.4), polyethylene glycol 4000 (9.1), propylene glycol (30.5), zinc stearate (5.0)
5. Pluronic base: Pluronic F-127 (22.0), distilled water (78.0)

Drug-containing ointments were prepared in 22% Pluronic base under stirring at 4°C, and preserved at room temperature for gelation.

Table 1
Scores of CEES-induced skin lesions

Score	Lesions
0.0	Normal
0.5	Bleaching
1.0	Petechiae
2.0	Mild purpura
3.0	Severe purpura
4.0	Mild ulcer
5.0	Severe ulcer

2.3. Application of 2-chloroethylethyl sulfide and ointments

Female hairless mice (body weight 25 ± 3 g) were anesthesized with pentobarbital sodium (35 mg/kg body weight i.p.), and the back side was wiped with a 70% ethanol swap. A 3M Magic adhesive tape with 2 holes (5 mm in diameter) was attached to the back side by pressing with a thumb over the marginal line of the holes. A drop (0.6 μl each) of 2-chloroethylethyl sulfide was applied in tape holes, and spread evenly over the uncovered skin of mice. The mice were kept for 5 min in a fume hood until there was complete evaporation of the solution. Ointment bases or drug-containing ointments were applied over the exposed skin, and the tape was removed 1 h after 2-chloroethylethyl sulfide exposure. Thereafter, the ointment was applied twice daily throughout the experiment.

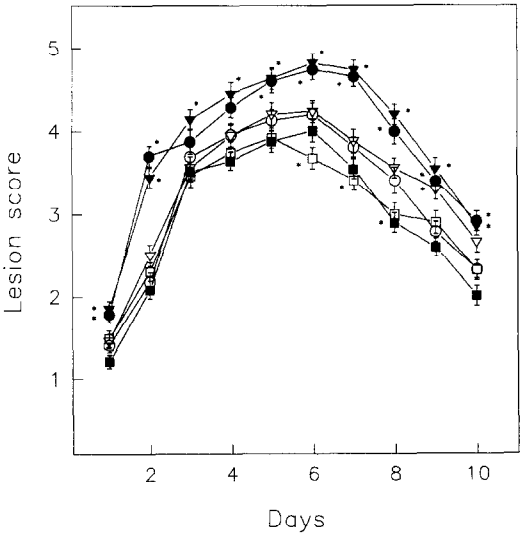

Fig. 1. Effects of ointment bases on the skin lesions induced by 2-chloroethylethyl sulfide. The back skin of hairless mice was exposed to 2-chloroethylethyl sulfide (0.6 μl/5 mm in diameter), and covered with each base 5 min after 2-chloroethylethyl sulfide exposure, and thereafter twice daily ($n = 20$ from 10 mice). ○, control (2-chloroethylethyl sulfide alone); ●, hydrophobic base; ▽, w/o base; ▼, o/w base; □, polyethylene glycol base; ■, Pluronic base. * Significantly different from control (2-chloroethylethyl sulfide alone) ($P < 0.05$).

Table 2
Mean shedding time of scabs

Bases	Days (mean ± S.E.M.)
2-Chloroethylethyl sulfide alone	9.52 ± 0.35
Hydrophobic base	10.58 ± 0.38 [a]
W/o base	10.46 ± 0.40
O/w base	9.69 ± 0.47
Polyethylene glycol base	9.17 ± 0.32
Pluronic base	9.26 ± 0.27

Each ointment base was prepared as described in Section 2 and applied as described in Fig. 1. The shedding time of each scab was recorded.
[a] Significantly different from control (2-chloroethylethyl sulfide alone) ($P < 0.05$).

2.4. Gross and microscopic examinations

Skin lesions were scored daily according to the criteria described in Table 1, and the change of body weight was recorded. Skin samples were fixed with 10% neutral formalin, embedded in paraffin and stained with hematoxylin & eosin for light microscopic examination. Data of gross examinations, lesion score and scab-shedding time, were expressed as the mean ± S.E.M. of 20 skin lesions (from 10 mice per group). Tests of significance were performed using unpaired Student's t-test with $P < 0.05$ as a criterion of difference.

The experiments reported here were conducted according to the Guide for Care and Use of Laboratory Animals (1985), as prepared by the Committee on Care and Use of Laboratory Animals, National Research Council USA, NIH Publ. No. 85-23.

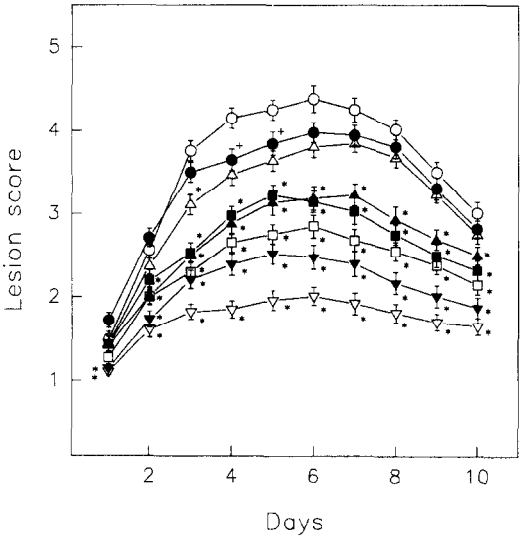

Fig. 2. Effects of ointments containing calmodulin antagonists or anesthetics on the skin lesions induced by 2-chloroethylethyl sulfide. Each drug was prepared in Pluronic base and applied as described in Fig. 1. ○, control (2-chloroethylethyl sulfide alone); ●, Pluronic base; ▽, 1% trifluoperazine; ▼, 2% thioridazine. □, 10% pentamidine; ■, 20% lidocaine; △, 10% ketoconazole; ▲, 5% pentobarbital. [+] Significantly different from control (2-chloroethylethyl sulfide alone) ($P < 0.05$); [*] Significantly different from Pluronic base ($P < 0.05$).

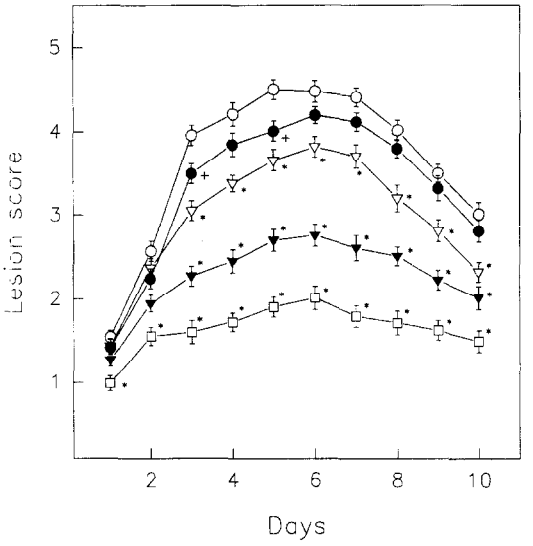

Fig. 3. Effects of trifluoperazine ointments of various concentrations on the skin lesions induced by 2-chloroethylethyl sulfide. ○, none (2-chloroethylethyl sulfide alone); ●, Pluronic base; ▽, 0.2% trifluoperazine; △, 0.5% trifluoperazine; □, 1.0% trifluoperazine. [+] Significantly different from control (2-chloroethylethyl sulfide alone) ($P < 0.05$); [*] Significantly different from Pluronic base ($P < 0.05$).

3. Results

Exposure of hairless mouse skin to 2-chloroethylethyl sulfide led to a time-dependent development of skin lesions up to 6 days (Fig. 1), with mice showing mild to moderate petechiae on the 1st day and an ulceration with a

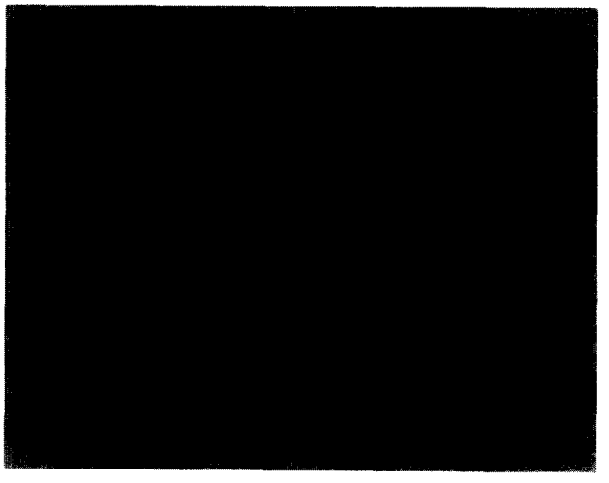

Fig. 4. Effects of trifluoperazine ointments on the skin lesions 3 days after 2-chloroethylethyl sulfide exposure. Note the thin, scaly scabs in the trifluoperazine-treated mice compared to the thick, reddish brown scabs with severe ulcer in the untreated mice. 1, none (2-chloroethylethyl sulfide alone); 2, Pluronic base; 3, 0.5% trifluoperazine; 4, 1.0% trifluoperazine.

thick scab after 3 days (Vogt et al., 1984). In general, the healing process started after 6 days, and the mean shedding time of scabs was 9.52 days (Table 2).

As shown in Fig. 1, oily bases, hydrophobic and o/w bases, markedly worsened the skin lesions and delayed the shedding of scabs. In contrast, water-soluble bases, such as polyethylene glycol, and Pluronic bases alleviated the lesions to some extent, and moreover, these bases advanced the shedding time. Based on these results, in the following studies we selected 22% Pluronic F-127 as a base for the drug-containing ointments.

Both trifluoperazine and thioridazine, derivatives of phenothiazine, substantially prevented the development of skin lesions induced by 2-chloroethylethyl sulfide exposure (Fig. 2), whereas phenothiazine itself failed to prevent the lesions, but rather promoted the development of ulcer. Another type of calmodulin antagonist, pentamidine, also exhibited a good protection, though it was less effective

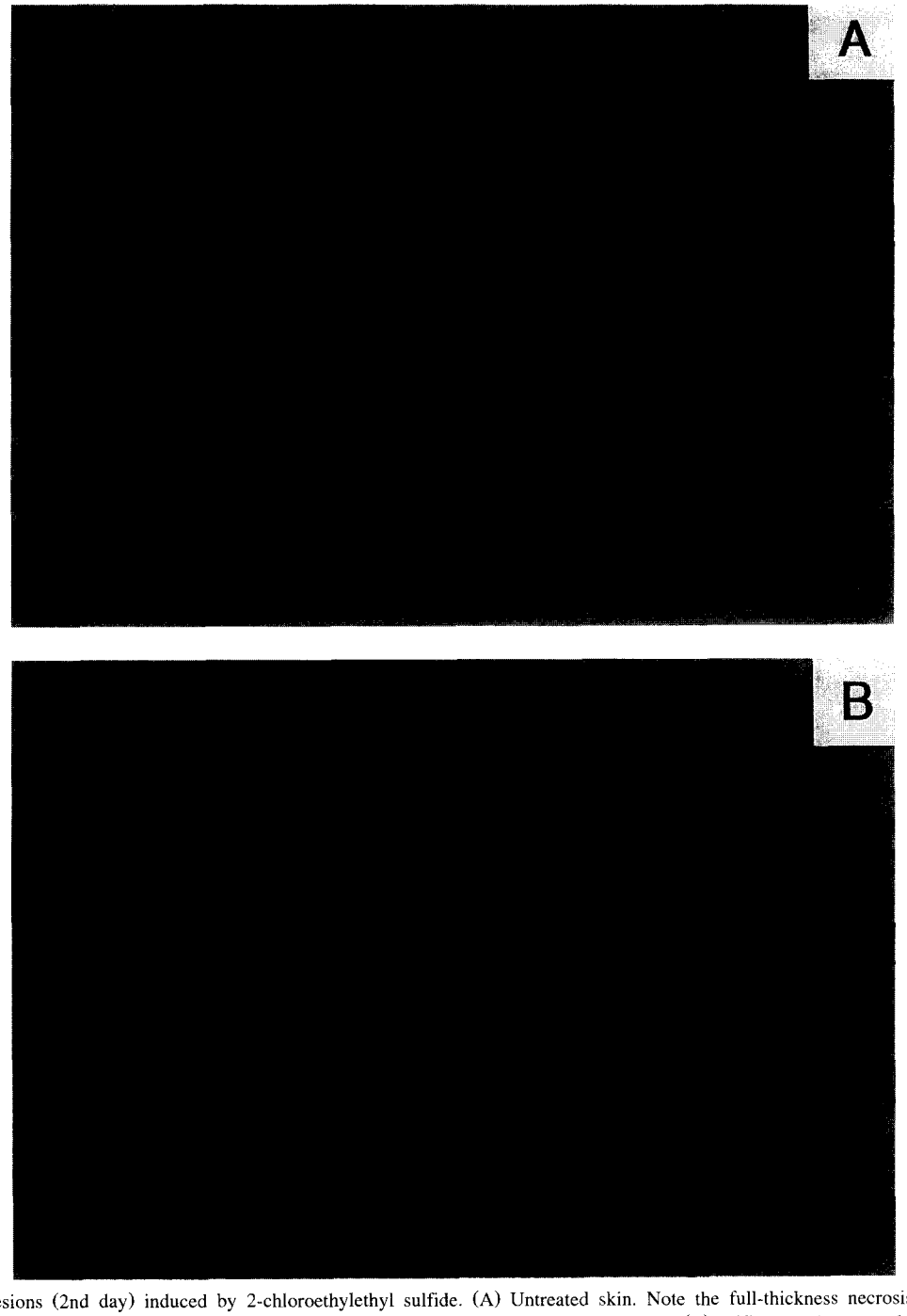

Fig. 5. Early skin lesions (2nd day) induced by 2-chloroethylethyl sulfide. (A) Untreated skin. Note the full-thickness necrosis, showing extensive hemorrhage and inflammatory cell infiltration, eventually leading to a compact scab deep in the dermis. (B) Trifluoperazine-treated skin. Note the 'silent cell death', with lesions showing vacuolization and nuclear fractionation or lysis.

than trifluoperazine. Ketoconazole, a weak calmodulin antagonist (Hegemann et al., 1993), provided much less protection. In addition, anesthetics were tested for their ability to prevent 2-chloroethylethyl sulfide-induced skin damage. Lidocaine, a local anesthetic, at high concentration (20%) afforded protection, whereas tetracaine and butacaine were less effective. Also, a marked protection was achieved with pentobarbital (5%). As shown in Fig. 3, the effective concentration ranges were > 0.2% for trifluoperazine, > 0.5% for thioridazine, > 5% for pentamidine, > 10% for lidocaine and > 2% for pentobarbital, suggesting that phenothiazine-type tranquilizers may be more beneficial for the treatment of 2-chloroethylethyl sulfide-induced burn than anesthetics. In a separate experiment, it was found that a high dose of trifluoperazine (> 1%) caused emaciation, leading to a decrease in body

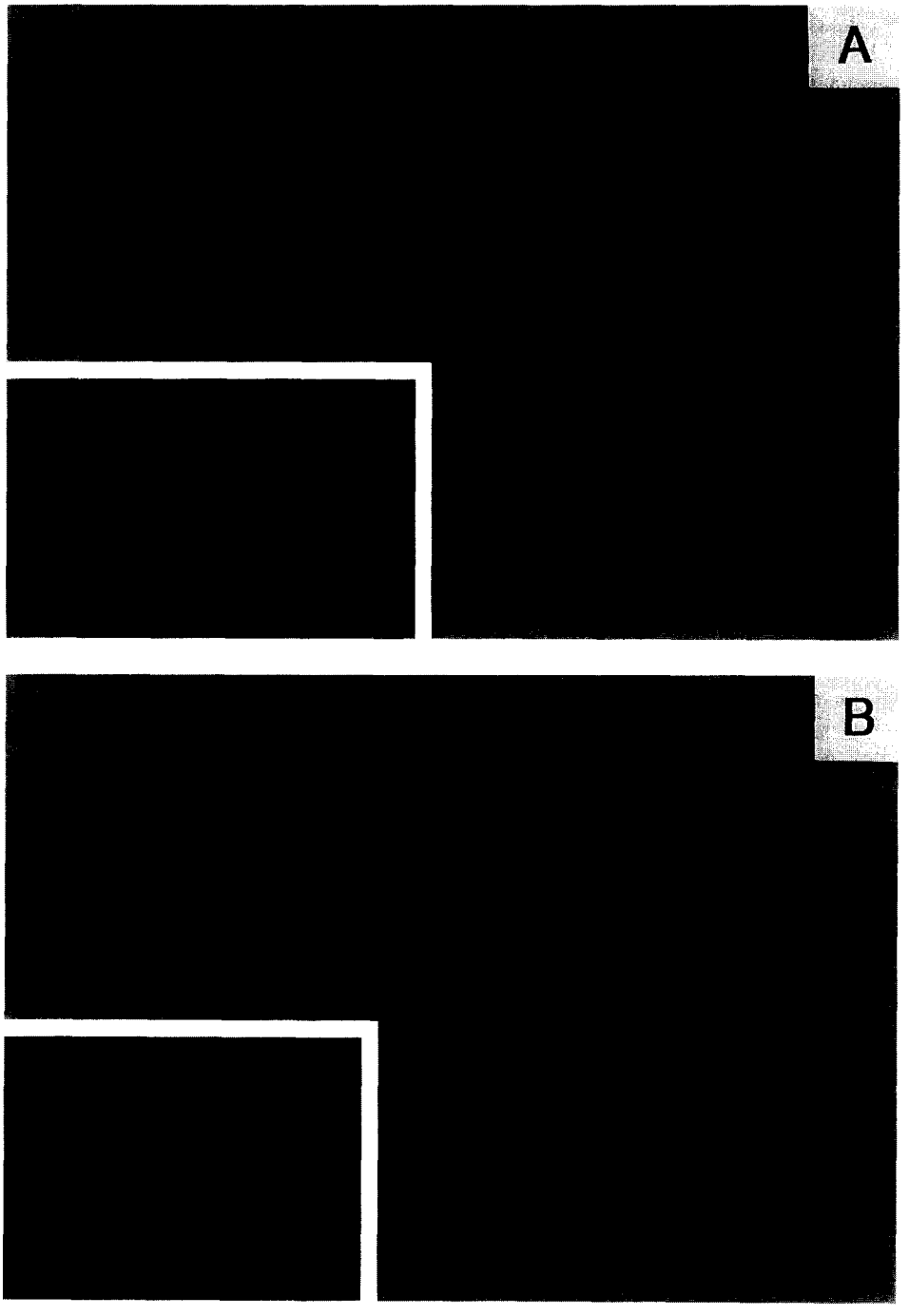

Fig. 6. Late skin lesions induced by 2-chloroethylethyl sulfide. (A) Untreated skin, showing a thick scab containing a large number of inflammatory cells (5th day). Inset: excessive proliferation of epithelial cells arround the scab from the normal skin in healing process (7th day). (B) Trifluoperazine-treated skin, showing the similar pattern of cell death to that of the 2nd-day lesions (5th day). Inset: extensive reepithelization from the viable follicular cells that survived the 2-chloroethylethyl sulfide exposure (7th day).

weight, and then a gradual recovery after 4 days (data not shown). Such a loss of body weight was rapidly recovered when the treatment was ceased, implying an anorexic condition after exposure to high doses of trifluoperazine. In contrast, pentamidine at high doses (5–10%) caused no remarkable decrease in body weight.

Mouse skin treated with trifluoperazine ointment showed bleaching and keratosis on earlier days, and scaly or light brown scabs without ulcer on later days, in contrast to purpura and resulting reddish brown scabs with a severe ulcer in the untreated (2-chloroethylethyl sulfide-exposed) skin (Fig. 4). 2-Chloroethylethyl sulfide-exposed skin without the subsequent treatment showed a full-thickness necrosis of affected area, resulting in extensive hemorrhage and infiltration of inflammatory cells, mainly neutrophils and macrophages, on the 2nd day (Fig. 5A). In contrast, Fig. 5B demonstrates that hemorrhagic and inflammatory changes were markedly reduced by 1% trifluoperazine therapy, although the necrosis of basal cells, showing vacuolization and nuclear fractionation or lysis (Nakamura et al., 1990; Mol et al., 1991), was not prevented. It was commonly observed that the formation of a thick scab (Fig. 6A) deep to the skin exposed to 2-chloroethylethyl sulfide disturbed the normal reepithelization from surrounding normal epidermis, which resulted in excessive proliferation arround the scab and in the formation of granulomatous scars in healing lesions (Fig. 6A, Inset). However, it was noteworthy that a typical pattern of 'silent cell death' in trifluoperazine-treated skin after exposure to 2-chloroethylethyl sulfide led to the formation of a thin scab, around which the excessive proliferation of epithelial cells was not observed (Fig. 6B). It is also of note that an active regeneration of keratinocytes from viable hair follicles was observed on the 7th day (Fig. 6B, Inset), while such viable hair follicles were not observed in the control skin exposed to 2-chloroethylethyl sulfide only.

4. Discussion

Although several mechanisms for the cytotoxicity of alkylating mustards have been proposed, and a number of protections against cytotoxicity have been reported (Meier and Johnson, 1992; Clayson et al., 1993; Choi et al., 1994, 1995), the mechanism of skin toxicity has not been clearly explained until now. Earlier skin tests (Vogt et al., 1984; Dannenberg et al., 1985; Harada et al., 1985, 1987) showed that the features of mustard-induced skin inflammations were somewhat different from those of mustard cytotoxicity. Rather, the pathogenesis of skin lesions induced by mustards were found to be very similar to that of thermal burn (Vogt et al., 1984; Requena et al., 1988; Yourick et al., 1993). Recently, it was proposed that the alkylation of basement membrane components, such as laminin, fibronectin and heparan sulfate proteoglycan, destabilizes dermal-epidermal junctions in the process of mustard-induced vesication (Monteiro-Riviere and Inman, 1995; Zhang et al., 1995). It seemed that a higher dose of mustard and a shorter exposure time were required for the skin lesions than for DNA alkylation (Mol and De Vries-Van de Ruit, 1992). Moreover, many other DNA-alkylating agents did not induce vesication and the other pathological changes observed with mustards (Papirmeister, 1993). Thus, DNA alkylation may not be a primary biochemical event in skin injury caused by mustards.

It would be a better strategy to prevent vascular leakage and promote reepithelization than to rescue the keratinocytes, the DNA of which had aleady been alkylated. This is well supported by our observation that calmodulin antagonists effective in the treatment of thermal burns, which is characterized by vascular leakage, successfully prevented 2-chloroethylethyl sulfide skin toxicity. However, it should be considered that most mustard on the skin evaporates, as suggested in the earlier reports (Somani and Babu, 1989). Therefore, a careful selection of ointment bases is important, because oily bases may prevent the remaining mustard from evaporating, thereby promoting its absorption through the skin and eventually leading to worsening the skin lesions. This might explain why in our study these oily bases prolonged the shedding time of scabs, implying a delay in the healing process.

Recent reports (Beitner et al., 1989b,1991) demonstrated that several calmodulin antagonists blocked thermal damage by inhibiting Ca^{2+}-phospholipase A_2 and glucose 1,6-bisphosphatase, which are calmodulin dependent. The protective action of trifluoperazine and thioridazine against 2-chloroethylethyl sulfide-induced burns might be partially explained by the inhibitory effect of these calmodulin antagonists on calmodulin-dependent enzymes including Ca^{2+}-phospholipase A_2. In further support of this, pentamidine, a calmodulin antagonist (Kitamura et al., 1995), prevented the skin lesions induced by 2-chloroethylethyl sulfide. However, calmodulin antagonists of phenothiazine derivatives shows systemic toxicity, in proportion to their potency to inhibit calmodulin action by inhibiting protein kinase C and antagonizing the dopamine receptor (Beitner et al., 1991). The loss of body weight and reduction of efficacy seen after application of trifluoperazine at high concentrations (> 1%) might be due to its non-selective actions.

Local anesthetics, such as lidocaine, tetracaine and butacaine, have been used for burn relief in various forms, especially in burn sprays. These local anesthetics are known to be inhibitors of Ca^{2+}-phospholipase A_2. Although some commercial burn sprays containing high concentrations (20%) of lidocaine or benzocaine, in 70% polyethylene glycol 400 containing 10% isopropyl alcohol, were not effective for the prevention of mustard skin toxicity, our study showed that all four anesthetics used in 22% Pluronic F-127 exerted a considerable protection at high concentrations. The lack of efficacy of commercial sprays might be due to deleterious effect of alcoholic components on the

skin lesions induced by 2-chloroethylethyl sulfide as observed with o/w base (Fig. 1). The beneficial effect of pentobarbital might be explained by the findings that pentobarbital-anesthesized mice showed less or delayed responses to the 2-chloroethylethyl sulfide-induced edema formation compared with non-anesthesized or ether-anesthesized animals, and that the vascular responses in pentobarbital-anesthesized mice started when the animal awoke from anesthesia (data not shown). Moreover, continuous action of pentobarbital, topically applied, attenuated the skin lesions induced by 2-chloroethylethyl sulfide, implying that the early vascular responses may govern the delayed ulcerative changes.

Although the exact mechanism of action of the calmodulin antagonists or anesthetics used remains to be clarified in further ultrastructural studies, our histopathologic findings suggest that these compounds have some actions on the vascular responses to chemical burns as inferred from the prevention of hemorrhage and inflammatory cell infiltration. In further support of our observations, trifluoperazine effectively prevented the edema formation caused by diphenylcyclopropenone, another well-known vesicant (unpublished data). Taken together, it is proposed that calmodulin antagonists, trifluoperazine, thioridazine and pentamidine, or anesthetics, lidocaine and pentobarbital, in water-soluble ointment bases might be a promising choice for the prophylactic and therapeutic treatments of 2-chloroethylethyl sulfide-induced skin burns.

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